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Immunomodulatory potential of extracts, fractions and pure compounds from *Phyllanthus amarus* and *Psidium guajava* on striped catfish (*Pangasianodon hypophthalmus*) head kidney leukocytes

Truong Quynh Nhu[a,b], Nguyen Phuc Dam[c,d], Bui Thi Bich Hang[b], Le Thi Bach[e], Do Thi Thanh Huong[b], Bui Thi Buu Hue[e], Marie-Louise Scippo[f], Nguyen Thanh Phuong[b], Joëlle Quetin-Leclercq[d], Patrick Kestemont[a,*]

[a] Research Unit in Environmental and Evolutionary Biology (URBE), Institute of Life, Earth and Environment (ILEE), University of Namur, Rue de Bruxelles 61, B-5000, Namur, Belgium
[b] College of Aquaculture and Fisheries, Cantho University, Campus II, Cantho City, Viet Nam
[c] Department of Chemistry Education, School of Education, Can Tho University, Can Tho City, Viet Nam
[d] Louvain Drug Research Institute (LDRI) Pharmacognosy Research Group, Université Catholique de Louvain, B-1200, Brussels, Belgium
[e] Department of Chemistry, College of Natural Sciences, Can Tho University, Can Tho City, Viet Nam
[f] Department of Food Sciences, Laboratory of Food Analysis, Faculty of Veterinary Medicine, Fundamental and Applied Research for Animals & Health (FARAH), Veterinary Public Health, University of Liège, Bât. B43bis, 10 Avenue de Cureghem, Sart-Tilman, Liège, Belgium

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ABSTRACT

This study aimed to identify major phytochemical constituents, as well as compare the immunomodulatory effects of *Psidium guajava* L. and *Phyllanthus amarus* Schun and Thonn crude ethanol extracts and their fractions on striped catfish (*Pangasianodon hypophthalmus*) head kidney leukocytes (HKLs). Moreover, pure constituents were also investigated for their effects on those cells: hypophyllanthin, identified as a major constituent of *P. amarus* crude extracts and its hexane fraction; corosolic acid, ursolic acid, and oleanolic acid, identified in *P. guajava* crude extract, ethyl acetate and dichloromethane fractions; with other terpenic derivatives, as well as guajaverin and avicularin, identified with other flavonoids by LC-UV-MS in the crude *P. guajava* extract and its ethyl acetate fraction. Cell viability, respiratory burst assay (RBA), nitric oxide synthase (NOS) and lysozyme activity in HKLs were analyzed after 24 h stimulation with each extract (10, 20 and 40 μg/mL) or pure compound (7.5, 15 and 30 μM). Our results show that the hexane fraction of both plant extracts inhibited the viability of HKLs, while several other fractions enhanced the cell viability. All *P. guajava* fractions at all or some concentration considerably enhanced the RBA production in HKLs. Similarly, NOS production was also significantly increased by some or all concentrations of *P. guajava* dichloromethane and ethyl acetate fractions. However, the NOS production was dose-dependently inhibited in HKLs treated with Pa ethyl acetate and both plants aqueous fractions at 10 or 10 and 40 μg/mL respectively. The lysozyme activity in cells treated with *P. guajava* crude extracts and all its organic solvent fractions were stronger than those in *P. amarus* treatments. Pure compounds including corosolic acid, guajaverin, ursolic acid, hypophyllanthin inhibited the HKLs viability according to concentration and type of compound. All pure compounds except avicularin significantly stimulated, at certain or all concentrations, the RBA production and/or the lysozyme activity in HKLs. The NOS production was significantly reduced in HKLs treated with oleanolic acid (30 μM) and hypophyllanthin (7.5 μM) while its level was increased by hypophyllanthin at 30 μM. These results highlighted that the crude ethanol extracts of *P. guajava* and *P. amarus*, their fractions and some of their pure components at certain concentrations can potentially act as immunomodulators, and could be considered as valuable candidates in fishery sciences.

* Corresponding author. Research Unit in Environmental and Evolutionary Biology, Institute of Life, Earth & Environment (ILEE), University of Namur, rue de Bruxelles 61, B-5000, Namur, Belgium.

E-mail addresses: tqnhu@ctu.edu.vn (T.Q. Nhu), npdam@ctu.edu.vn (N.P. Dam), btbhang@ctu.edu.vn (B.T. Bich Hang), ltbach@ctu.edu.vn (L.T. Bach), dtthuong@ctu.edu.vn (D.T. Thanh Huong), btbhue@ctu.edu.vn (B.T. Buu Hue), mlscippo@ulg.ac.be (M.-L. Scippo), ntphuong@ctu.edu.vn (N.T. Phuong), joelle.leclercq@uclouvain.be (J. Quetin-Leclercq), patrick.kestemont@unamur.be (P. Kestemont).

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1. Introduction

Strengthening immunity is one of the promising strategies to control infectious diseases in aquaculture. In aquatic animals, the innate immunity is mainly developed, probably in compensation to the rather limited adaptive immunity when compared to higher vertebrates [1,2]. Mononuclear phagocytes including monocytes and macrophages are the key players in the immune system and other cellular processes such as lymphocyte proliferation, inflammatory and apoptosis [3,4]. To destroy and engulf foreign pathogens, macrophages will release antibacterial substances including reactive oxygen species (ROS), which are usually measured by respiratory burst assay, and reactive nitrogen species via phagocytosis process [5,6]. Ulvestad et al. (2018) reported that both immunostimulants including bacterial lipopolysaccharide (LPS) and ß-glucan could stimulate the ROS production in Atlantic salmon (*Salmo salar*) macrophages [7]. Similarly, the ROS activity was also increased in Longfin yellowtail *Seriola rivoliana* leukocytes treated with phenolic compounds from hawthorn (*Crataegus mexicana*) nanoencapsulated with maltodextrin [8]. Monocytes and macrophages also play a vital role in producing lysozyme, although the latter was recorded with a higher level of lysozyme [9]. It was indicated that tumor necrosis factor-α (TNF-α) could regulate the production of lysozyme in monocytes and macrophages, while neutrophils are responsible for the release of lysozyme [10,11].

Natural products have been applied as a promising source for medicinal discovery, because they may be effective to control various diseases and mitigate many side effects that are associated with synthetic drugs. Among plants of potential medical interest, guava (*Psidium guajava* L.) and bhumi amla (*Phyllantus amarus* Schum and Thonn) are known to possess pharmacological activities including anti-bacterial, anti-oxidant as well as immune response activities. *P. guajava* leaf extract significantly improved the growth performance, mucosal and serum immunity as well as antioxidant activities in rohu (*Labeo rohita*) or Mozambique tilapia (*Oreochromis mossambicus*) [12–14]. Indeed, the guava leaf extract could stimulate the production of NOS and ROS in common carp *Cyprinus carpio* var. *koi* L [15], RBA and lysozyme activity in rohu [14]; whereas the mRNA expression of *iNOS* significantly decreased in rohu fed with guava extract [12]. Moreover, several *in vitro* studies also demonstrated that *P. amarus* and *P. guajava* extracts attenuated LPS induced proinflammatory response in RAW 264.7 cells [16] and U937 macrophages [17], respectively. Additionally, flavonoids isolated from *P. guajava* as well as phyltetralin, niranthin and nirtetralin identified from *P. amarus* were also revealed to possess anti-inflammatory activity via the inhibition of LPS induced cytokines such as TNFα, IL1β, IL10, iNOS, and COX-2 [18,19]. Ilangkovan et al. (2013) also suggested that phyllanthin and hypophyllantin strongly suppressed phagocytic activity in human leucocytes [20]. In additon, after a large *in vitro* screening of 20 commonly used traditional plant extracts (using striped catfish leukocyte models isolated from blood and head kidney) followed by an *in vivo* validation, we previously found that *P. amarus* and *P. guajava* ethanol extracts potentially modulated immune endpoints and better protected striped catfish against the infection by the bacteria *Edwardsiella ictaluri* [21–23].

Nowaday, plant-based natural sources are rapidly developing as commercial products due to the increasing demand for sustainable applications in aquaculture. Hence, new insights on chemical characterization as well as analysis of their biological activities are necessary to improve our knowledge, standardize active extracts and activities, and determine the mode of action/indications. Our earlier studies only focused on the immunomodulatory effects of *P. amarus* and *P. guajava* crude ethanol extracts on the striped catfish. In the present study, to try to identify the type of compound(s) which could contribute to this activity, we fractionated these crude extracts with increasing polarity solvents and separated them in fractions containing different concentrations and types of bioactive compounds (from less polar to more polar compounds) [24]. As the crude extract of *P. amarus* contained a large amount of tannins, which can have unspecific effects on cells [25], we removed these tannins on a polyamide column to obtain the *P. amarus* non-tannin fraction. Finally, these fractions were investigated for their immunomodulatory effects to get a deeper understanding about which kind of metabolites can influence these activities. In the present study, some phytochemical constituents of the *P. amarus* and *P. guajava* crude ethanol extracts and fractions will be then identified. Moreover, the immunomodulatory effects of these crude extracts, their fractions (n-hexane, dichloromethane, ethyl acetate, water and non-tannin), and pure compounds were investigated using striped catfish head kidney leukocyte model.

2. Material and methods

2.1. Chemicals

All organic solvents (VWR, Belgium) *n*-hexane, dichloromethane, ethyl acetate, methanol, ethanol used for sample extraction and fractionation were of analytical grade. Methanol, acetonitrile, formic acid were HPLC grade and water had MilliQ quality. The standards of ellagic acid, hyperin, isoquercitrin, guajaverin, avicularin, asiatic acid, corosolic acid, maslinic acid, oleanolic acid, ursolic acid were purchased form Sigma (Belgium), while hypophyllanthin was obtained from Extrasynthese (Genay – France). The mixture of four triterpenic ester (4TTE) including 3*β*-O-(*cis-p*-coumaroyl)corosolic acid, 3*β*-O-(*trans-p*-coumaroyl)corosolic acid, 3*β*-O-(*cis-p*-coumaroyl)maslinic acid, and 3*β*-O-(*trans-p*-coumaroyl)maslinic acid were isolated by Lucy Catteau as explained by Ref. [26] and the purity of this mixture was measured as 93% using an Accela HPLC system (Thermo Fisher Scientific). Polyamide CC6 powder was purchased from Macherey-Nagel GmbH (Duren, Germany) and used for the elimination of tannins.

2.2. Plant material

Leaves of *P. guajava* were collected at Binh-Minh district, Vinh-Long province, Vietnam and aerial parts of *P. amarus* were collected at Thot-Not district, Can-Tho city, Vietnam and identified by PhD. DANG Minh Quan (Department of Biology Education, School of Education, Can Tho University). Voucher specimens of Psig2017, Phya2017, respectively were deposited at Department of chemistry, College of Natural Sciences, Can Tho University.

2.3. Preparation of plant extracts and fractions

The dry powders of *P. guajava* leaves and *P. amarus* aerial parts (1 kg) were macerated with ethanol (3 x 5 L) at room temperature during 24 h, then filtered by -paper filter(Whatman™ 1001-400 Grade 1 Qualitative Filter Paper, Diameter: 40 cm, Pore Size: 11 μm). The solutions were concentrated under reduced pressure with a rotatory evaporator at 45 °C until obtaining of a dark syrup which was then lyophilized to give 114 g (*P. guajava*) or 133 g (*P. amarus*) crude extracts. 530 mg *P. guajava* crude extract were suspended in water (60 mL) and fractionated using liquid-liquid partition three times with 20 mL of hexane, dichloromethane, and ethyl acetate to obtain 4 fractions: hexane (66 mg), dichloromethane (75 mg), ethyl acetate (94 mg) and aqueous fractions (210 mg), respectively. 6g of *P. amarus* crude extract was suspended in 700 ml of water and then fractionated using liquid-liquid partition with 3 x 250 mL of hexane and ethyl acetate to give 3 fractions: hexane (1 g), ethyl acetate (600 mg) and aqueous fractions (4 g), respectively.

Elimination of tannins of crude extract of *P. amarus*.

Twenty mg of crude *P. amarus* extract were suspended in water and applied on a 5 g polyamide column (h = 11 cm, Ø = 1 cm) in order to eliminate the tannins. The column was eluted with 10 mL water, 10 mL of 50% of MeOH/water and then 40 mL of MeOH. The obtained solutions were combined and evaporated under reduced pressure to furnish

13 mg of non-tannin fraction.

All samples were stored at 5 °C prior to be used for the immunomodulatory effects test and for identification of active compounds by HPLC-DAD-orbitrap-MS for *P. guajava* and HPLC-UV for *P. amarus*.

The extracts and fractions were re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) in order to prepare stock solutions at 8, 4 and 2 mg/mL for *in vitro* study. The stock solutions were stored at −20 °C until used.

2.4. Analysis of samples by HPLC-DAD-Orbitrap-MS

Analyzes were performed on an Accela HPLC system (Thermo Fisher Scientific) consisting of a PDA detector connected with a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer from the UCLouvain Massmet platform. HR-MS spectra were measured with APCI source in the negative mode using full-scan MS with a mass range of 100–2000 *m/z*. The following (−) APCI conditions were applied: vaporizer temperature, 400 °C; sheath gas (N_2) flow rate, 25 a.u.; auxiliary gas (N_2) flow rate, 25 a.u.; sweep gas (N_2) flow rate, 5 a.u.; capillary temperature, 250 °C; capillary voltage, 10 V; tube lens, 125 V.

Identification of phenolic compounds was done on a Phenomenex® Lichrospher C18, 4.6 × 250 mm column packed with 5 μm particles. 10 μL of samples at 1 mg/mL concentration were injected in the full loop injection mode. The column was eluted at a constant flow rate of 0.8 mL/min using a binary solvent system: solvent A, MilliQ water 0.1% formic acid and solvent B, acetonitrile HPLC grade (0–10 min, 17% B; 28 min, 28% B; 38 min, 38% B; 39–49 min, 100% B). DAD-UV detector was set at 254 nm.

The selected column for analysis of triterpenic acids was an Agilent® Poroshell 120 EC-C18, 4.6 × 100 mm packed with 2.7 μm particles. 20 μL of samples (1 mg/mL) were injected in the full loop injection mode. A flow rate of 0.4 mL/min was applied for this method and the mobile phase consisted of a gradient of solvent A, MilliQ water; solvent B, acetonitrile HPLC grade and solvent C, methanol HPLC grade (0–3 min, 35% A, 30% B and 35% C; 30–48 min, 0% A, 65% B and 35% C). DAD-UV detector was set at 210 nm and the LC-orbitrap conditions were the same as previously mentioned.

2.5. Analysis of samples by HPLC-UV

The crude *P. amarus* extract, as well as its fractions and pure hypophyllanthin were analyzed by HPLC-UV using the method described by Jantan et al. (2014) on a Hitachi Merck® equipment with a L6200 intelligent pump and UV L4000 Merck® detector. The column was a RP18 Licrospher® (5 mm, 250 mm × 4.5 mm). The flow rate was 0.4 mL/min. The mobile phase consisted of MilliQ water 0.1% orthophosphoric acid (A) and acetonitrile (B) (0 min, 5% B, 20–35 min, 95% B, 36–50 min, 100% B). UV detector was set at 205 nm.

2.6. Experimental fish

For *in vitro* experiments, a total of 80 striped catfish juveniles of 50 ± 5 g were acclimated to laboratory conditions for 15 days at 28 ± 2 °C in composite tank (2000 L). Fish were fed twice (9 a.m. and 3 p.m.) daily at a feeding rate of 2% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described in the previous study [21]. Healthy fish which did not present abnormal clinical and pathogenic bacteria, were used for experiment. Fish were randomly sampled and anesthetized by immersion in ethyl-aminobenzoic acid (MS222: 100 mg/L, Sigma Aldrich, USA). Then fishes were euthanized by cervical dislocation.

Table 1
List of extracts, fractions and pure compounds.

Name	EXPLANATION
Pg Extract	Crude extract of *Psidium guajava*
Pg-F1	Fraction *n*-hexane of *P. guajava*
Pg-F2	Fraction CH_2Cl_2 of *P. guajava*
Pg-F3	Fraction ethyl acetate of *P. guajava*
Pg-F4	Fraction H_2O of *P. guajava*
Pa Extract	Crude extract of *Phyllanthus amarus*
Pa-F1	Fraction *n*-hexane of *P. amarus*
Pa-F2	Fraction ethyl acetate of *P. amarus*
Pa-F3	Fraction H_2O of *P. amarus*
Pa-F4	Non tannin fraction of *P. amarus*
Avicularin- Avi	Found in crude extract of *P.guajava*
Guajaverin- Gua	Found in crude extract of *P. guajava*
Corosolic Acid- Cor	Found in crude extract of *P. guajava*
Ursolic Acid- Urs	Found in crude extract of *P. guajava*
Olenanolic Acid- Ole	Found in crude extract of *P. guajava*
Hypophyllanthin- Hyp	Found in crude extract of *P.s amarus*

2.7. Isolation of head kidney leukocytes (HKLs)

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-μm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 μg/mL streptomycin +10,000 U/mL penicillin (Invitrogen). After washing with PBS 1X, the residual erythrocytes in HKLs were removed by incubating in 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen). Viable cells were adjusted to 5×10^6 cells/mL after enumeration using trypan blue stain (VWR, Leuven, Belgium) and seeded in wells of a 24 or 48-well plate (Greiner Bio-One, Vilvoorde, Belgium).

2.8. Stimulation of primary HKLs

After isolation of striped catfish HKLs, 2 mL of cell suspension (5×10^6 cells/mL) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A were added to each well of 24-well plate (Greiner Bio-One, Vilvoorde, Belgium). The concentrations of extracts and fractions used in the current study were selected upon our previous trials. Depending on the first evaluation of the effects of 20 plant ethanol extracts [21], two plant extracts including *P. amarus* and *P. guajava* at 10 μg/mL strongly stimulated the humoral immune response in the peripheral blood mononuclear cells (PBMCs) and head kidney leukocytes (HKLs) of striped catfish. Moreover, another experiment was performed at 0; 10; 25; 50; 75; 100 μg/mL *P. guajava* ethanol extract, the results showed that *P. guajava* could strongly stimulate immune response in HKLs at 25 and 50 μg/ml. So, each crude extract or fraction was analyzed at the concentrations of 10, 20 and 40 μg/mL in the present study, to determine the best efficient ones.

The concentrations of pure compounds were selected in combination between bibliographic review and *in vitro* prior test realized with different concentrations of rutin (0; 0.75; 1.5; 3; 6 and 12 μM) in HKLs. Moreover, several publications reported that concentrations of several pure compounds usually ranged from 2.5 to 50 μM when added into mammal macrophages [27–32]. So, each pure compound at 7.5, 15, 30 μM was selected for *in vitro* study.

Afterward, leukocytes stimulation was carried out with at 10, 20 and 40 μg/mL for each crude extract and fraction or at 7.5, 15, and 30 μM for each pure compound (Table 1). Cells cultivated in the same

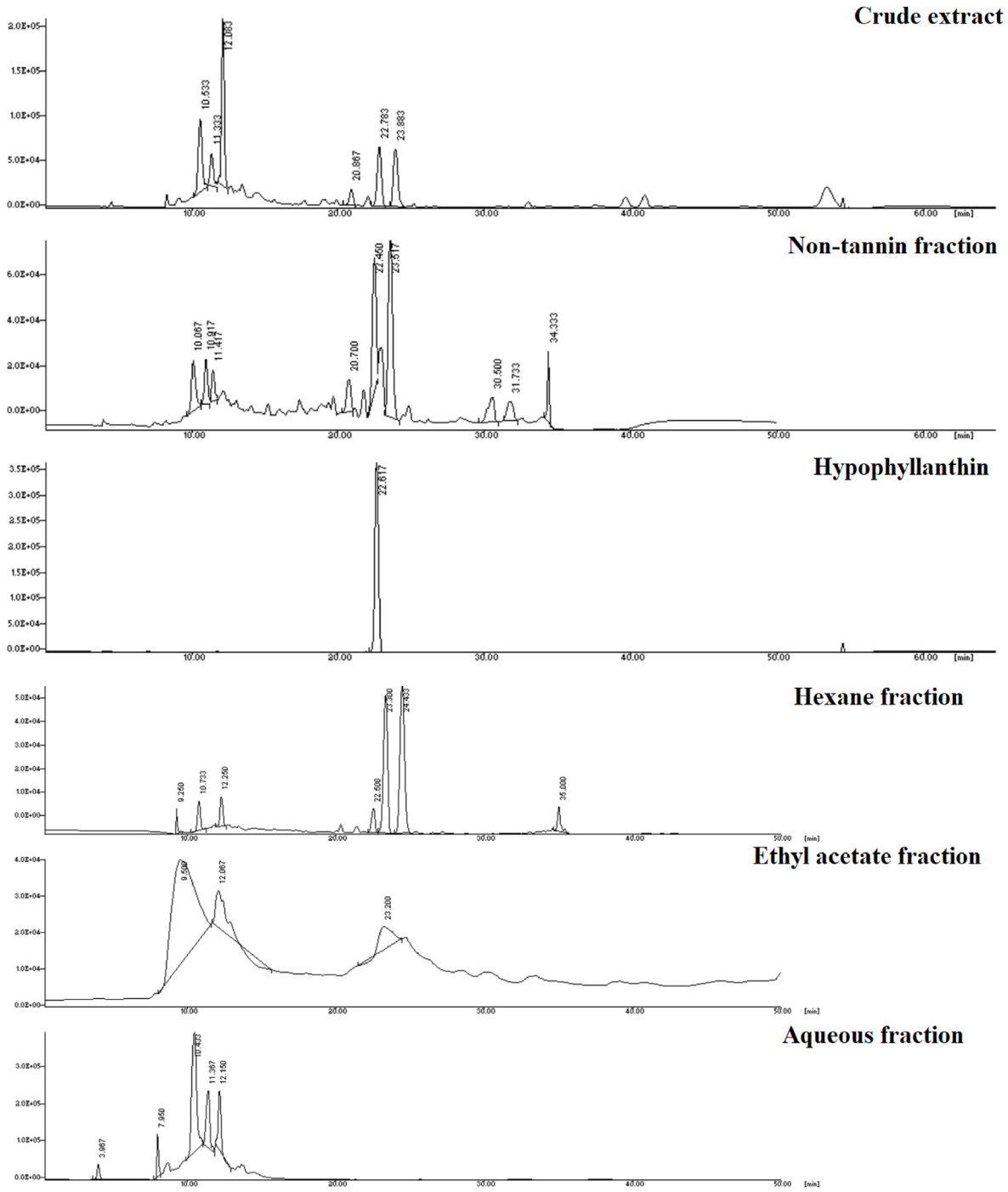

Fig. 1. HPLC-UV (205 nm) profiles of the crude *P. amarus* extracts with and without tannins, hypophyllanthin and hexane, ethyl acetate and water fractions from its crude ethanolic extract.

Table 2
Identification of phenolic compounds from *P. guajava* leaves using HPLC-DAD-QTOF-MS method.

Peak	Retention time (min)	λ_{max} (Metwally et al.)	Molecular ion (*m/z*) $[M - H]^-$	Mw	Formula	Error (ppm)	Compounds	Reference
1	26.91	254, 367	300.99899	302	$C_{14}H_6O_8$	1.096	Ellagic acid	[38,39]
2	27.30	255, 354	463.08868	464	$C_{21}H_{20}O_{12}$	1.578	Hyperin	[39]
3	27.83	256, 353	463.08810	464	$C_{21}H_{20}O_{12}$	0.998	Isoquercitrin	[38,39]
4	29.85	255, 353	433.07779	434	$C_{20}H_{18}O_{11}$	1.252	Reynoutrin	[38–40]
5	30.81	255, 353	433.07791	434	$C_{20}H_{18}O_{11}$	1.372	Guajaverin	[38–40]
6	31.87	255, 350	433.07681	434	$C_{20}H_{18}O_{11}$	0.272	Avicularin	[38,39]
7	32.76	255,350	301.03543	302	$C_{15}H_{10}O_7$	1.151	Unknown	

Table 3
Identification of triterpenic acid from *P. guajava* leaves using HPLC-DAD-QTOF-MS method.

Peak	Retention time (min)	λ_{max} (Metwally et al.)	Molecular ion (*m/z*) $[M-H]^-$	Mw	Formula	Error (ppm)	Compounds	Reference
8	13.81	203	487.34351	488	$C_{30}H_{48}O_5$	1.709	Asiatic acid	[41]
9	23.79	204	471.34830	472	$C_{30}H_{48}O_4$	1.414	Maslinic acid	[42]
10	24.47	205	471.34808	472	$C_{30}H_{48}O_4$	1.194	Corosolic acid	[41]
11	26.17	204, 307	617.38544	618	$C_{39}H_{54}O_6$	1.774	3*β*-O-(*cis/trans-p*-coumaroyl)maslinic acid	[43]
12	26.81	204, 307	617.38446	618	$C_{39}H_{54}O_6$	0.794	3*β*-O-(*cis/trans-p*-coumaroyl)corosolic acid	[43]
13	27.44	205, 311	617.38519	618	$C_{39}H_{54}O_6$	1.524	3*β*-O-(*trans/cis-p*-coumaroyl)maslinic acid	[43]
14	28.08	205, 311	617.38568	618	$C_{39}H_{54}O_6$	2.014	3*β*-O-(*trans/cis-p*-coumaroyl)corosolic acid	[43]
15	30.13	205	455.35333	456	$C_{30}H_{48}O_3$	1.358	Oleanolic acid	[41]
16	30.43	205	455.35397	456	$C_{30}H_{48}O_3$	1.998	Ursolic acid	[41]

medium containing 0.5% DMSO served as control. Each experiment was conducted in triplicates. The HKLs were incubated at 28 °C in a humidified atmosphere of 5% CO_2.

2.9. Viability test with extract fractions and pure compounds

Cell viability was determined after stimulation with different concentrations of extract, fractions and pure compounds. Each treatment was triplicates. The determination was done by a MTS test following the manufacturer's protocol. Briefly, 20 µL of MTS test reagent solution (CellTiter 96® Aqueous One Solution Reagent, Sigma, USA) was added to the cells in culture medium (100 µL) (after exposure to different doses of extracts, fractions, or pure compounds for 24 h in a 96-well plate); a measurement of absorbance at 490 nm was then carried out after 4 h of incubation at 28 °C. The cell viability was calculated by the percentage ratio between the absorbance of the treated and that of the cell control without extract, considered as 100%.

2.10. Immune variables

The humoral immune response was then assessed for 24 h post stimulation (hps) with the concentrations mentioned above. The cell suspension was collected for nitric oxide species and respiratory burst assays. The residual cell suspension was collected by centrifugation at 10000 × *g* at 4 °C in 5 min, the supernatant was used for lysozyme analysis.

2.10.1. Lysozyme assay

The lysozyme assay protocol was adapted from Ellis [33] and Milla et al. [34] for HKLs and serum of striped catfish. In 96-well microplates, the lysozyme activity assay was initiated by mixing 30 µL of cell suspension with 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, MO, USA) suspension in phosphate buffer, pH 6.2 (0.3 mg/mL). The difference in absorbance at 450 nm was monitored between 0 and 15 min and used to calculate units of lysozyme activity. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.10.2. Respiratory burst assay

Respiratory burst was adapted from Rook et al. [35]. HKLs were washed twice in L-15 medium (1000 g, 5 min, 28 °C). The culture media were then replaced by a corresponding fresh medium containing 2 mg ml^{-1} NBT. Cells were incubated during 1 h at 28 °C in a light protected environment. After 1h, the cells were washed twice in PBS and the reaction was stopped by adding 200 µL of methanol. The cells were rinsed by centrifugation (1000 g, 10 min, 4 °C) and finally air dried for 10 min. Resulting formazan was dissolved in 240 µL of KOH 2 M and 280 µL of N-dimethylformamide. The absorbance of the final supernatant was measured at 550 nm. A standard curve was done using serial dilutions of NBT directly dissolved in KOH 2 M and N-dimethylformamide. Samples and negative control (without cells) were performed in duplicates. The activity was reported on protein concentration in cell suspension measured by Bradford assay [36].

2.10.3. Nitric oxide species assay

Production of NOS was measured by the Griess reaction. First, 100 µL of cell suspension were incubated with 5 µL of *E. ictaluri* suspension (OD 2) resuspended in corresponding culture media during 1 h at 28 °C. Then, 100 µl of Griess reactant was added and solutions were incubated for 15 min. The absorbance was measured at 540 nm. Standard straight line was performed by using serial dilutions of $NaNO_3$. Negative control corresponded to culture media (without cells) incubated with *E. ictaluri* suspension and Griess reactant. The activity was reported on protein concentration in cell suspension measured by Bradford assay [36].

2.11. Statistical analysis

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY:IBM USA). Results are presented as mean ± standard deviation (S.D.). One-way analysis of variance was run to find out any difference in immune parameters in treated cells compared to control cells.

3. Results

3.1. Chemical constituent identification of P. amarus by HPLC-UV

The HPLC-UV profiles of the crude extract and fractions of *P. amarus* were analyzed according to Jantan et al. [37]. The chromatograms are given on Fig. 1. They show that crude extracts before and after removing tannins contain hypophyllathin, as well as the hexane fraction.

3.2. Chemical constituent identification of P. guajava by HPLC-DAD-Orbitrap-MS

To identify the major components of the crude ethanol extract of *P. guajava* collected in Vietnam, HPLC-DAD-Orbitrap-MS chromatographies using two different mobile phases were performed on this crude ethanol extract and its fractions. First, the phytochemical constituents of the crude extract were identified based on characteristic properties of the peaks in the chromatograms such as the retention times, UV absorption spectra, experimental mass data, best matching molecular formula provided by the Xcalibur software of the Orbitrap-MS fragments and comparison with the reported data of other samples of *P. guajava* leaves. Then, the presence of these compounds was confirmed by comparison with the HPLC chromatograms of reference compounds.

The results showed that two major groups of metabolites were present in the crude extract: phenolic and triterpenic compounds. Identified compounds are summarized in Table 2 and Table 3. The chromatograms of these two main bioactive groups are shown in Fig. 2

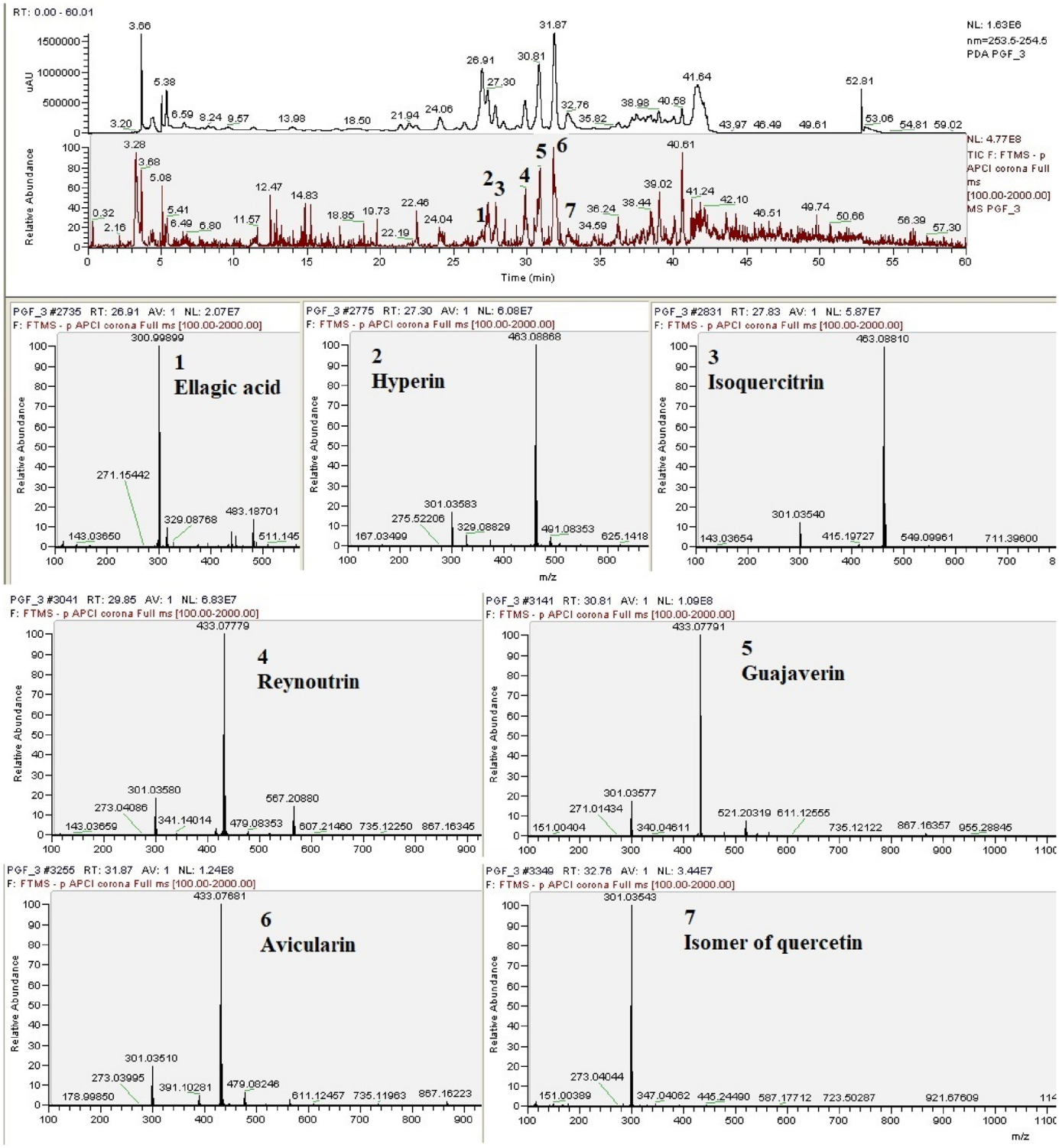

Fig. 2. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the crude ethanolic extract of *P. guajava* with the method used to analyse phenolic compounds and mass spectra of identified phenolic compounds in negative ion mode.

and Fig. 3 respectively.

The major phenolic compounds from the crude extract of *P. guajava* (see Figs. 2 and 4 and Table 2) were ellagic acid (compound **1**) [38,39], hyperin (compound **2**) [39], isoquercitrin (compound **3**) [38,39], guajaverin (compound **5**) [38–40], avicularin (compound **6**) [38,39]. However, there was not available standard for the compound **4**, which was an isomer of guajaverin and avicularin, and which showed the same UV spectra and MS molecular ion of $[M - H]^-$ 433.07779, corresponding to the formula $C_{20}H_{18}O_{11}$ and identified as reynoutrin in comparison with published data [38–40]. Compound **7** possessed the same mass fragments and UV spectrum but different retention times compared to quercetin or morin standards. It could suggest that the compound **7** was an isomer of quercetin. These phenolic compounds were already identified from other samples of leaves of *P. guajava* [38–40]. The structures of these compounds are given in Fig. 5. The results of Fig. 6 showed that these phenolic compounds were mainly

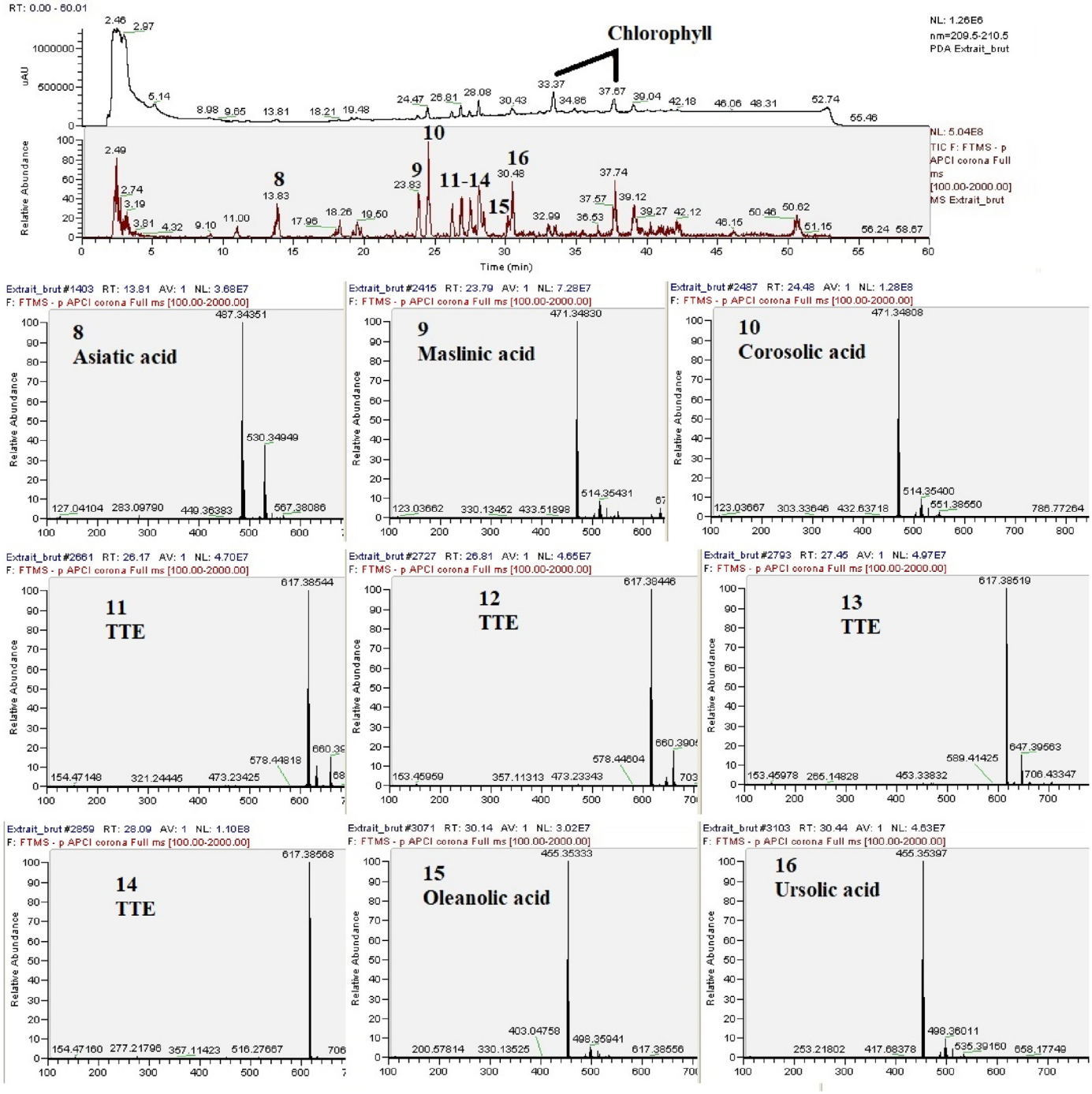

Fig. 3. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the crude ethanolic extract of *P. guajava* with the method used to analyse triterpenic compounds and mass spectra of identified triterpenic compounds in negative ion mode.

present in the ethyl acetate fraction.

The data from Figs. 3 and 7 and Table 3 proved that the major triterpenic derivatives from the leaves of *P. guajava* were asiatic acid (compound **8**) [41], maslinic acid (compound **9**) [42], corosolic acid (compound **10**) [41], 4TTE (3*β*-O-(*cis-p*-coumaroyl)corosolic acid, 3*β*-O-(*trans-p*-coumaroyl)corosolic acid, 3*β*-O-(*cis-p*-coumaroyl)maslinic acid, and 3*β*-O-(*trans-p*-coumaroyl)maslinic acid) (compounds **11–14**) [43], oleanolic acid (compound **15**) [44] and ursolic acid (compound **16**) [44]. These components were also previously isolated from other samples of this plant and reported in the literature [38–40]. Their structures were given in Fig. 8. The results of Fig. 9 indicated that these triterpenic compounds were present in both the dichloromethane and ethyl acetate fractions. Although these compounds were known and reported to be present in *P. guajava* in previous publications, this was the first time that an HPLC-DAD-MS method is described to identify these triterpenic derivatives in the leaves of *P. guajava*.

In conclusion, flavonoids and triterpenic derivatives were the major components identified in the crude ethanol extract from the leaves of *P. guajava*, its dichloromethane fraction mainly contained triterpenic derivatives as major metabolites and its ethyl acetate fraction contained both phenolic and triperpenic derivatives.

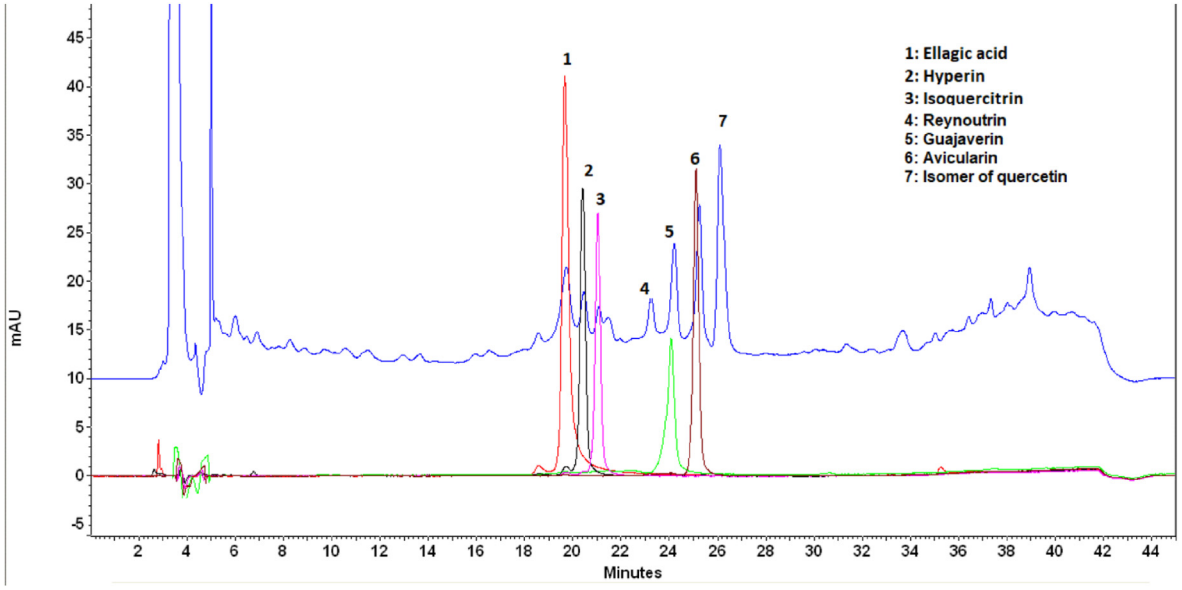

Fig. 4. HPLC-DAD-UV chromatograms of crude ethanolic extract of *P. guajava* with the method used to analyse phenolic compounds and the standards of ellagic acid, hyperin, isoquercitrin, guajaverin and avicularin at 254 nm.

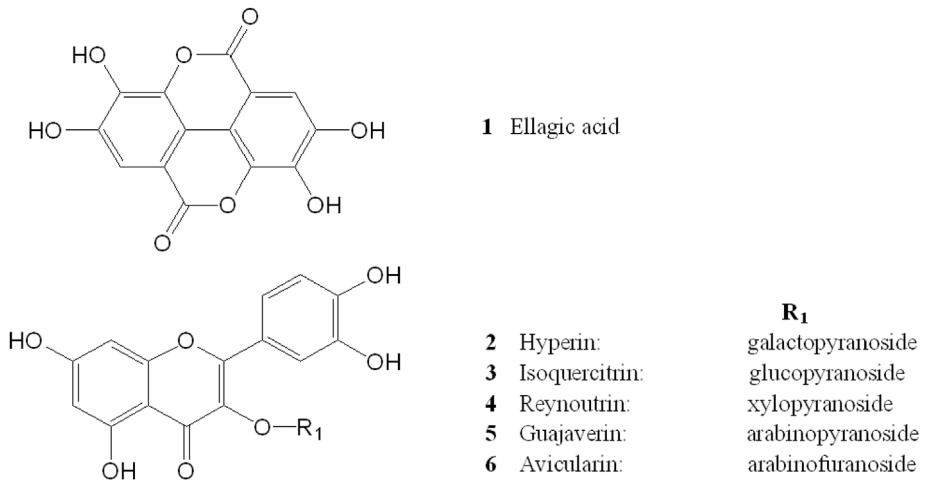

Fig. 5. Structures of phenolic compounds identified form *P. guajava* leaves.

3.3. Effects of extract fractions on immune response of HKLs

3.3.1. Viability

After stimulation, all tested concentrations of *P. amarus* and *P. guajava* crude extracts as well as *P. amarus* non-tannins and *P. guajava* aqueous fractions did not significantly affect the viability of striped catfish HKLs after 24 h (Fig. 10). The viability was significantly reduced in cells treated with *P. guajava n*-hexane (all concentrations) and *P. amarus n*-hexane fractions (40 μg/mL) compared to controls. In contrast, high and medium concentrations of *P. amarus* ethyl acetate and aqueous fractions showed higher viability than controls HKLs. Similarly, HKLs growth was also stimulated with the highest concentration of *P. guajava* dichloromethane and ethyl acetate fractions after 24 h treatment. Nevertheless, the percentage of remaining cells was always higher than 75%, even for the highest concentrations, showing the low toxicity of these extracts.

3.3.2. Immune parameters

As shown in Fig. 11, the RBA, NOS and lysozyme levels in HKLs treated with most *P. guajava* fractions were increased compared to those treated by *P. amarus* fractions.

All concentrations of *P. guajava* dichloromethane fraction significantly enhanced the RBA production in HKLs after 24 h contact, while only medium and low concentrations of *P. guajava n*-hexane fraction, and the lowest concentration of *P. guajava* ethyl acetate and aqueous fractions could significantly increase the RBA levels in those cells. Crude *P. amarus* and *P. guajava* extracts and all *P. amarus* fractions did not affect the RBA activity in HKLs after 24 h.

Similarly, all concentrations of crude *P. amarus* and *P. guajava* extracts, *P. amarus n*-hexane, *P. amarus* non-tannins and *P. guajava n*-hexane fractions did not influence to the NOS production, whereas all concentrations of *P. guajava* dichloromethane and ethyl acetate fractions significantly increased the NOS level in HKLs compared to control after 24 h. However, the NOS production was significantly suppressed in HKLs treated with high and low concentrations of *P. amarus* ethyl acetate and *P. guajava* aqueous fractions, as well as with the lowest concentration of *P. amarus* aqueous fraction.

Most of the extract fractions differentially modulated the lysozyme activity in HKLs in a concentration dependent manner. Specifically, all concentrations of *P. guajava n*-hexane, dichloromethane and ethyl acetate fractions significantly stimulated the lysozyme activity in HKLs after 24 h. Several concentrations of *P. guajava* crude extract (highest

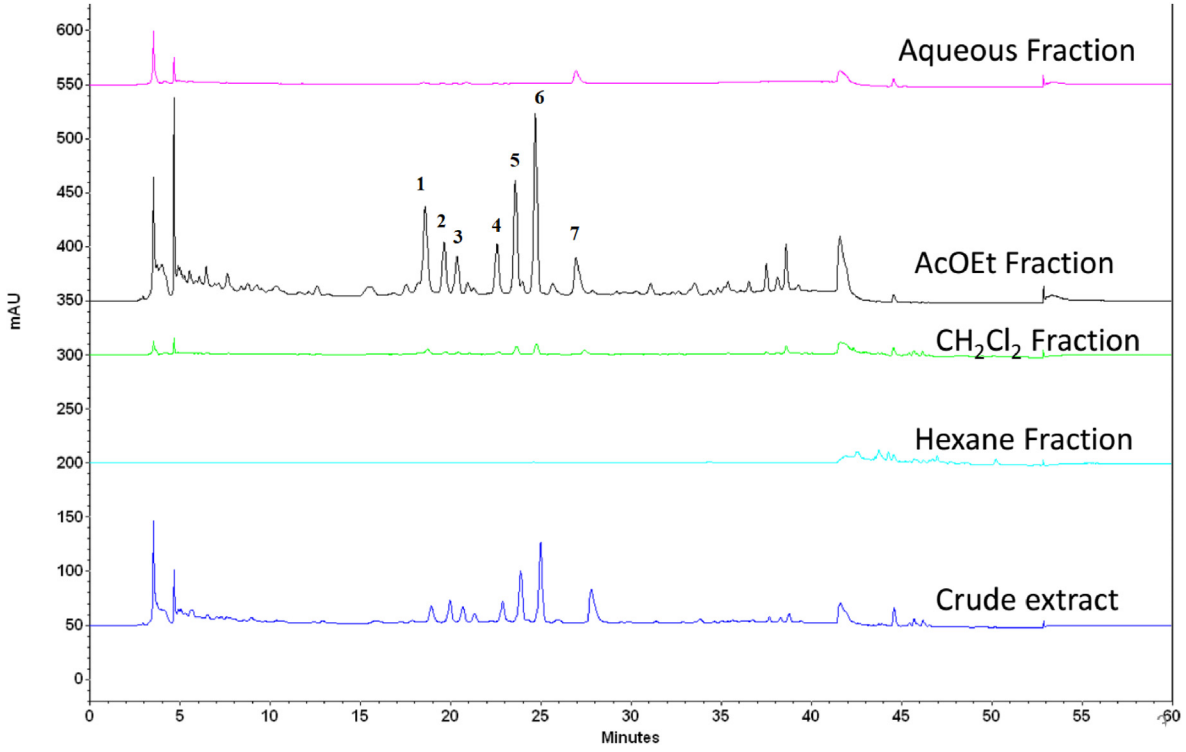

Fig. 6. HPLC-DAD-UV chromatograms of the crude ethanolic extract of *P. guajava* and its four fractions with the method used to analyse phenolic compounds at 254 nm.

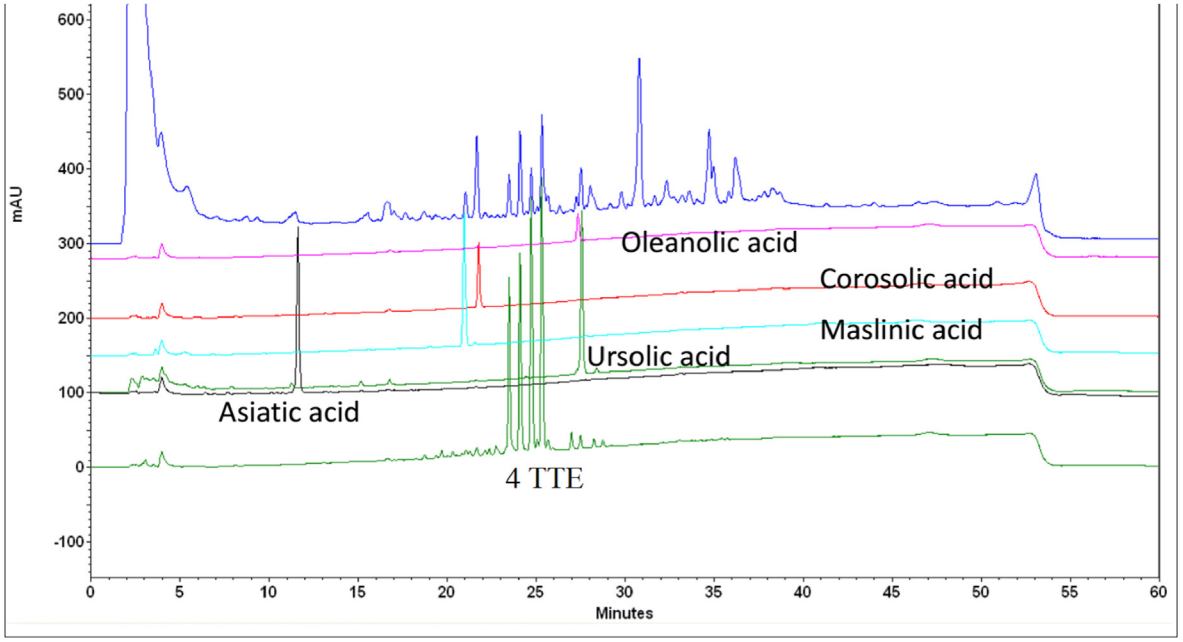

Fig. 7. HPLC-DAD-UV chromatograms with the method used to analyse triterpenic compounds of the crude ethanolic extract of *P. guajava* and the standards of asiatic acid, maslinic acid, corosolic acid, 4TTE, oleanolic acid and ursolic acid at 210 nm.

and lowest) and *P. amarus* fractions including aqueous (medium and lowest), and non-tannins (highest and medium) also significantly enhanced the lysozyme level in HKLs. Moreover, the *P. amarus* crude extract, *n*-hexane, ethyl acetate and *P. guajava* aqueous fractions did not affect the lysozyme activity in HKLs at 24 h.

3.4. Effects of pure compounds on immune response of HKLs

3.4.1. Viability

After 24 h, all concentrations of corosolic acid and ursolic acid significantly reduced the viability of HKLs compared to control ($p < 0.001$) (Fig. 12). Moreover, the highest and medium concentrations of guajaverin, and medium concentration of hypophyllanthin also significantly affected the viability of HKLs. However, the viability of HKLs was not affected by avicularin and oleanolic acid stimulation.

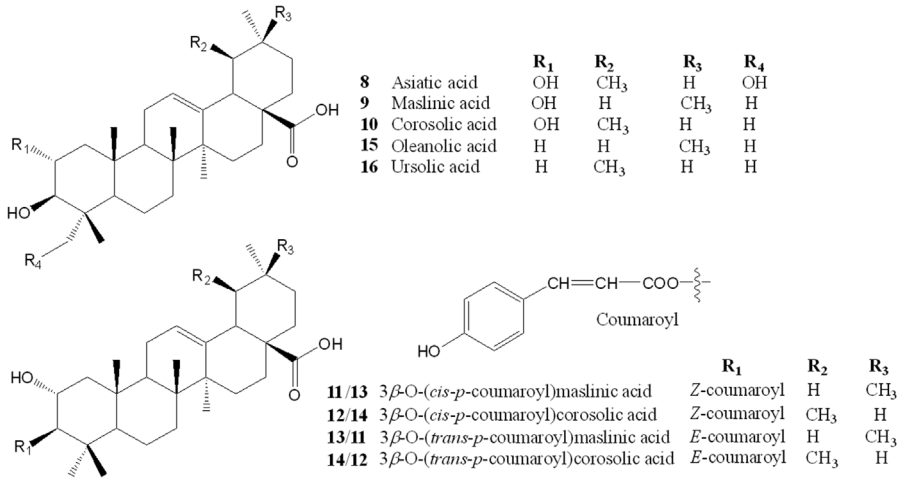

Fig. 8. Structures of triterpenic compounds identified form *P. guajava* leaves.

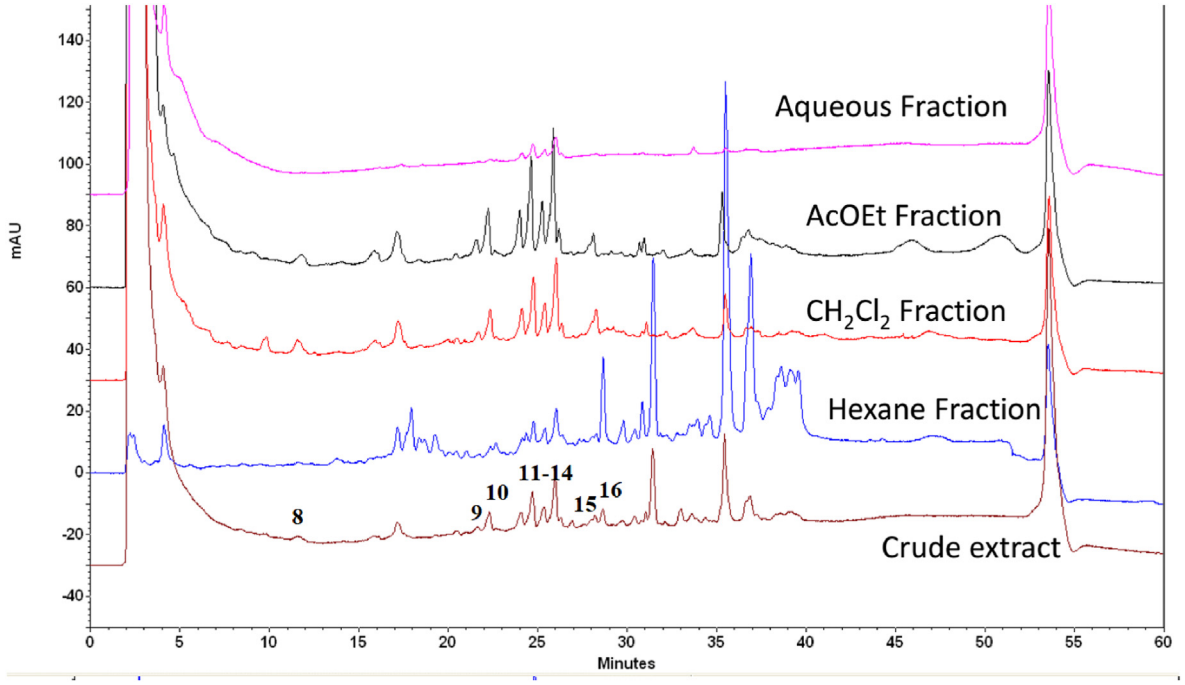

Fig. 9. HPLC-DAD-UV chromatograms of the crude ethanolic extract of *P. guajava* and its four fractions with the method of used to analyse triterpenic compounds at 210 nm.

Nevertheless, the percentage of remaining cells was always higher than 75%, even for the highest concentrations.

3.4.2. Immune parameters

The production of RBA was significantly increased in HKLs treated with all concentrations of guajaverin ($p < 0.001$) (Fig. 13). Moreover, HKLs treated with corosolic acid (7.5 μM), oleanolic acid (15 μM) and ursolic acid (30 μM) significantly enhanced their RBA level after 24h ($p < 0.05$). On the other hand, RBA was not affected by avicularin and hypophyllanthin.

After 24h stimulation, the highest concentration of hypophyllanthin significantly enhanced the NOS production, while the level was significantly decreased in HKLs treated with the lowest dose of hypophyllanthin and the highest dose of oleanolic acid ($p < 0.001$). Other pure compounds including avicularin, corosolic acid, guajaverin and ursolic acid did not affect NOS activity in striped HKLs.

With regards to humoral immune response, hypophyllanthin (all concentrations), avicularin (15 μM), guajaverin (7.5 μM), oleanolic acid (30 μM), corosolic acid (30 μM) and ursolic acid (30 μM) significantly increased the lysozyme activity in HKLs after 24 h contact, whereas only ursolic acid (7.5 μM) inhibited the lysozyme activity ($p < 0.05$).

4. Discussion

Medicinal plants, as well as their components, are well known as alternative natural resources, acting as antimicrobial, anti-stress, growth promotion, appetite stimulation, and immunomodulatory agents [45]. In aquaculture, these plant products are usually applied to activate immune responses including phagocytic cells, humoral immune response (lysozyme, complement, total immunoglobulin, peroxidase), cellular immune response (RBA, NOS), as well as inflammatory or anti-inflammatory responses, with the ultimate goal to protect fish from infectious pathogens [46]. Two solvents including ethanol and methanol are commonly used for plant extraction process. The different

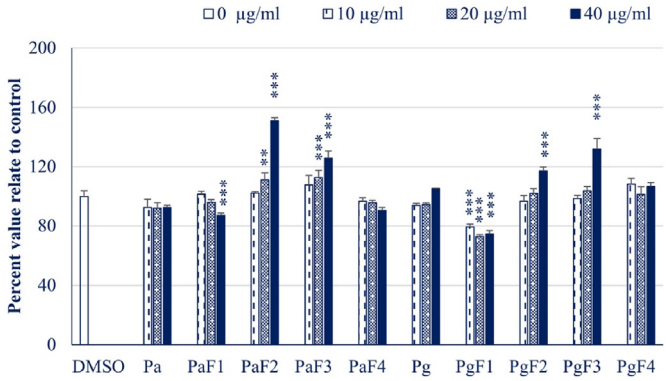

Fig. 10. Effect of crude ethanolic extracts of *P. amarus* and *P. guajava* and their fractions on viability of striped catfish HKLs after 24h. Pa: *P. amarus*; Pg: *P. guajava*; n-hexane- PaF1 and PgF1; dichloromethane-PgF2; ethyl acetate- PaF2 and PgF3; aqueous- PaF3 and PgF4; and non-tannin fraction- PaF4. Asterisk indicates significant differences in viability levels between stimulated and unstimulated cells at 24h (*$p < 0.05$; **$p < 0.01$; *** < 0.001). Values are mean ± S.D. (n = 3).

polarities of the extracting solvents may cause a wide variation in the level of bioactive compounds [24], which may lead to different effective degrees on aquatic animal immune responses. In the present study, we assessed the immunomodulatory effects of crude *P. guajava* and *P. amarus* ethanol extracts and their fractions including n-hexane, dichloromethane, ethyl acetate, and aqueous for *P. guajava* and n-hexane, ethyl acetate, aqueous, and non-tannin for *P. amarus* on striped catfish head kidney leukocytes. Moreover, major phytochemical constituents of the crude ethanol extracts (*P. amarus* and *P. guajava*) were identified and examined for their contributions on immune responses of striped catfish head kidney leukocytes.

Our study highlighted that *P. guajava* crude ethanol extract mainly contained flavonoids and triterpenic derivatives. Phenolic and triperpenic derivatives were the major components of *P. guajava* ethyl acetate fraction, while only triterpenoids were identified in its dichloromethane fraction. It could be concluded that the polarity of the solvents affected the phytochemical compositions of plant extract fractions. Moreover, variations in chemical compositions may arise in the same plant species according to the different habitats, geographical locations, seasons, and parts of the plant [47,48]. In contrast with our results (plant collected in Viet Nam), *P. guajava* collected in Gampéla (Burkina Faso, West Africa) and its ethanol leaves extract mainly contained anthocyans, alkaloids, flavonoids, tannins and steroids/terpenoids (triterpenoids), while only alkaloids and steroids/terpenoids were isolated by dichloromethane extract [49].

In the case of *P. amarus* crude ethanol extract from the plant collected in the Mekong delta of Vietnam, the HPLC-UV qualitative determination indicated that hypophyllantin was one of the major compounds before and after removing tannins, as well as its hexane fraction. Similarly, *P. amarus* collected in Indonesia contained a high amount of hypophyllantin, whereas *P. amarus* from Malaysia contained a higher amount of phyllanthin [37]. This outcome could suggested that the variations in the chemical compositions may be responsible for the different biological activity of the extract/fractions.

In the present study, the viability of striped catfish HKLs was differentially affected according to the dose and type of extract/fraction and compound. Indeed, the *n*-hexane fractions of *P. guajava* (40, 20 and 10 µg/mL) and *P. amarus* (40 µg/mL) were cytotoxic to HKLs after 24 h of stimulation. In line with these results, Kim et al. (2017) also indicated that the viability of raw 264.7 macrophages decreased after stimulation with *n*-hexane fraction (50 µg/mL) of marsh labrador tea *Ledum palustre* L. at 24 h [50]. However, *n*-hexane fraction from *Nerium oleander* L. flower significantly stimulated the proliferation of raw 264.7 macrophages followed by lipopolysaccharide stimulation (100 µg/mL) [51]. Our results also highlighted that the dichloromethane and ethyl acetate fractions of *P. guajava*, the ethyl acetate and aqueous fractions of *P. amarus* could enhance the HKL proliferation after 24 h of stimulation/contact. Cell viability was increased in a concentration dependent manner. It was already mentioned in the literature that at various concentrations from 0.1 to 10 µg/mL, chloroform, ethyl acetate and aqueous fractions of *Gynura procumbens* ethanol leaf extract significantly stimulated the proliferative effect in raw 264.7 macrophages after 24 h [52]. In addition, the pure compounds may affect the viability of striped catfish HKLs in a type and/or dose dependent manners. To the best our knowledge, publications about the effects of these pure compounds on leukocytes or macrophages of animals do not exist so far. Several studies have demonstrated the significant reduction in the viability of human retinoblastoma Y-79 stimulated with corosolic acid at concentrations from 0.5 to 20 µM [53] and in renal carcinoma Caki cells treated with 5 and 10 µM of corosolic acid [53,54]. Moreover, the viability in HepG2-human hepatoma and Caco2-human epithelial colorectal adenocarcinoma cell lines were significantly decreased after stimulated with mixture of oleanlic/ursolic acid at 32 µM [55]. The present results also showed that the treatment of 15 µM (about 6.5 µg/mL) hypophyllanthin significantly reduced the viability at 24h, but not other concentrations. However, Jantan et al. showed that the viability of human polymorphonuclear leukocytes and monocytes were always higher than 95% after stimulated with increasing concentrations from 0.3125 to 5 µg/mL of hypophyllanthin [37].

Phagocytosis is one of the first steps in the stimulation of the immune response and inflammation [56]. Moreover, ROS are mainly released during phagocytosis [57], and commonly measured by respiratory burst assay [58]. In fish, RBA has been applied as an indicator of innate immune responses [59–61]. In the current study, *P. amarus* and its fractions did not affect the RBA production in HKLs. Fractions from *P. guajava* including *n*-hexane (20 and 10 µg/mL), dichloromethane (40, 20 and 10 µg/mL), ethyl acetate (10 µg/mL), and aqueous (10 µg/mL) significantly induced the RBA in HKLs after 24 h contact. It could be postulated that *P. guajava* extract fractions showed a dose-dependent stimulatory effect on RBA via the activation of NADPH oxidase enzyme in HKLs in HKLs [62]. A similar increase of ROS production was observed in Mozambique tilapia fed with *P. guajava* ethanol extracts for 30 days [63]. Methanol extract of *P. amarus* hairy root induced the apoptosis in MCF7- cells via the increase of ROS level [64]. The present study also indicated that pure compounds isolated from crude ethanol extracts of *P. guajava* including corosolic acid (7.5 µM), guajaverin (30, 15 and 7.5 µM), oleanolic acid (15 µM), and ursolic acid (30 µM) significantly increased the RBA production in HKLs. In agreement with our results, López-García et al. (2015) also demonstrated that triterpenes including oleanolic acid and ursolic acid at 0.625 µg/mL (about 1.4 µM) or 2.5 µg/mL (about 5.5 µM) stimulated the production of ROS in the early phase of mouse macrophage J774 A.1 cell line infected with *Mycobacterium tuberculosis* [27]. Murine peritoneal macrophages treated with ursolic acid at 4 or 20 µM increased the ROS generation and then released IL-1β protein via the ATP-binding cassette transporter [28]. Moreover, guajaverin is a quercetin derivative [29]. In parasites (*Leishmania amazonensis*) infected macrophages, quercetin at 50 µM [30], 100 µM [31], or 3–12 µM [32] could stimulate the increase of ROS production after 72 h and this is in accordance with the increase of ROS production in HKLs treated with guajaverin.

Similar to RBA activity, nitric oxide mediates many physiological and pathophysiological processes including inflammation, and is released via macrophages activation and responsible for killing pathogens [65]. We presently found that dichloromethane and ethyl acetate fractions from *P. guajava* extract significantly enhanced the NOS production in striped catfish HKLs at 24 h. The NOS level was also increased in tilapia fed *P. guajava* ethanol extract [66]. However, the NOS production was significantly reduced in HKLs treated with aqueous fraction (40 and 10 µg/mL) from *P. guajava* extract, ethyl acetate (40

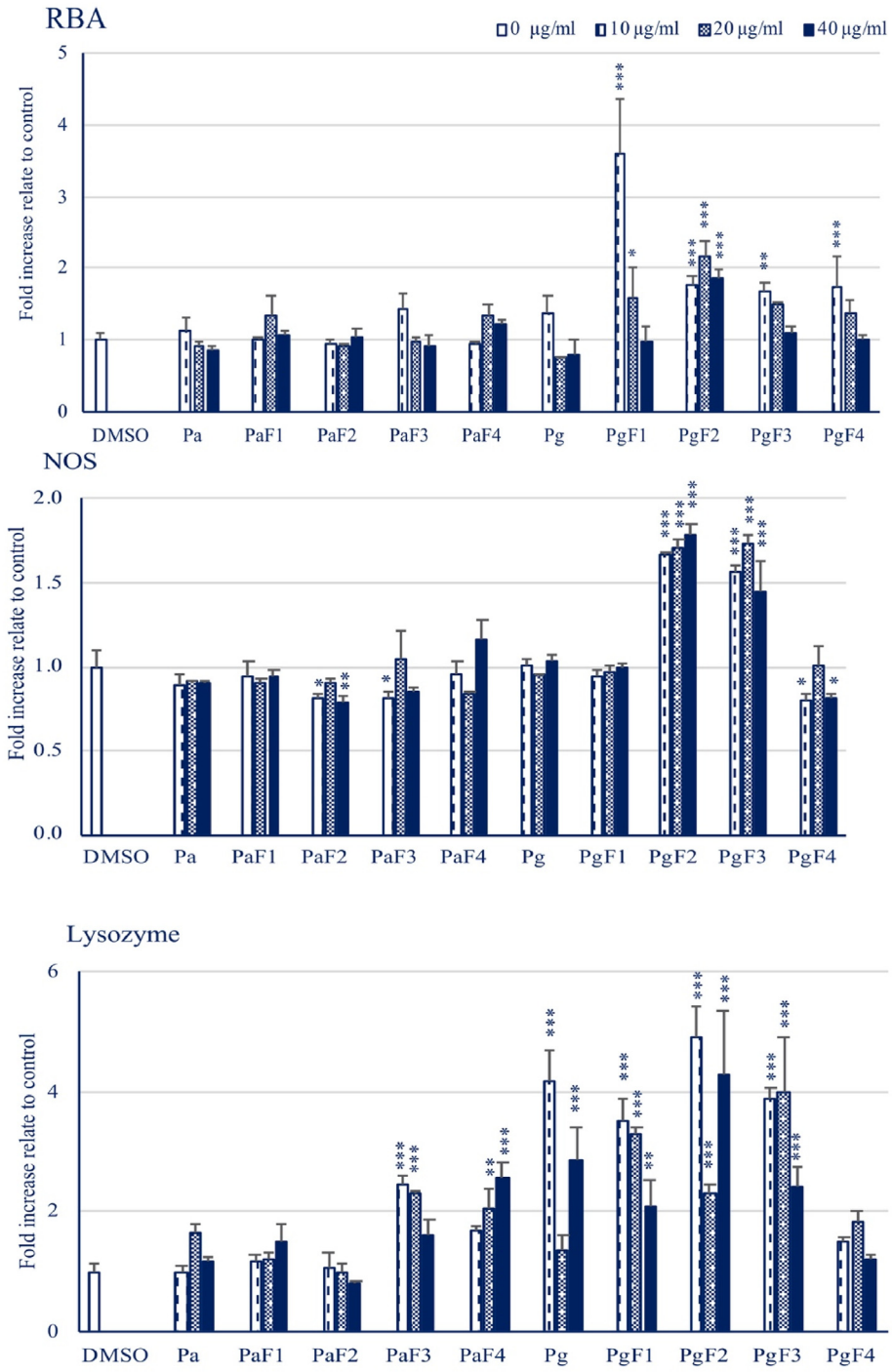

Fig. 11. Effect of crude ethanolic extracts of *P. amarus* and *P. guajava* and their fractions on RBA, NOS and lysozyme activities in striped catfish HKLs at 24h. Pa: *P. amarus*; Pg: *P. guajava*;n-hexane- PaF1 and PgF1; dichloromethane-PgF2; ethyl acetate- PaF2 and PgF3; aqueous- PaF3 and PgF4; and non-tannin fraction- PaF4. Asterisk indicates significant differences in lysozyme levels between stimulated and non-stimulated cells at 24h (*$p < 0.05$; **$p < 0.01$; *** < 0.001). Values are mean ± S.D. (n = 3).

and 10 μg/mL) and aqueous (10 μg/mL) fractions from *P. amarus* extract. This is in accordance with previous publications showing that *P. amarus* extract and its components possess inhibitory effects on NOS production in human phagocytes and neutrophils [20,67]. The ethanol/water and hexane *P. amarus* extracts at different doses also reduced the iNOS and COX-2 levels in mice stimulated with LPS [68]. The increased levels of NOS production in HKLs treated with *P. guajava* extracts, dichloromethane and ethyl actetate fractions may be due to the presence of flavonoids and triterpenic derivatives. Oleanolic acid (30 μM) and hypophyllanthin (7.5 μM) significantly inhibited the NOS production, while corosolic acid, guajaverin, avicularin and ursolic acid did not affect NOS production in HKLs. On the contrary, oleanolic acid at 0.625 μg/mL (about 1.4 μM) or 2.5 μg/mL (about 5.5 μM) was shown to enhance NOS production in J774 A.1 cells infected with *M. tuberculosis* [27]. In RAW 264.7 macrophages, oleanolic acid at 5, 10 and 50 μM was reported to possess antitumor activities, and could exhibit a dose dependent increase in *iNOS* and tumor necrosis factor- α transcripts by activating nuclear factor-κB [69].

With regard to lysozyme activity, we found that the lysozyme levels in HKLs stimulated by the *P. guajava* ethanol extract and its fractions

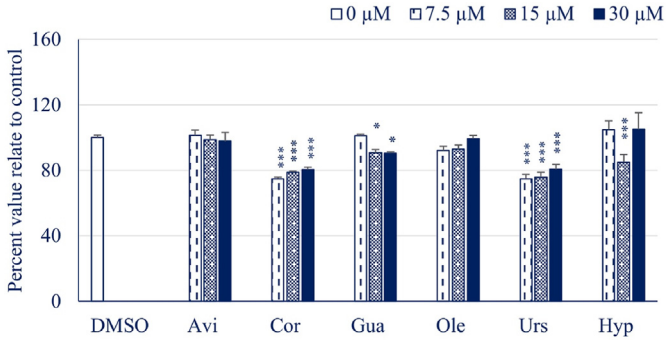

Fig. 12. Effect of pure compounds isolated from ethanolic extracts of *P. amarus* and *P. guajava* on viability of striped catfish HKLs at 24h. Cor: corosolic acid; Gua: guajaverin; Urs: ursolic acid; Hyp: hypophyllanthin; Avi: avicularin and Ole: oleanolic acid. Asterisk indicates significant differences in lysozyme levels between stimulated and non-stimulated cells at 24h (*$p < 0.05$; **$p < 0.01$; *** < 0.001). Values are mean ± S.D. (n = 3).

(except aqueous fraction) were usually stronger than those stimulated by *P. amarus* ethanol extract and its fractions. In consistence with these results, we previously also found that the lysozyme activity in HKLs and peripheral blood mononuclear cells (PBMCs) treated with *P. guajava* ethanol extract was considerably enhanced in a dose dependent manner, while *P. amarus* ethanol extract did not affect the lysozyme activity in striped catfish HKLs after 24 h [21]. Lysozyme plays a vital role in fish innate immune response and is mainly produced by macrophages [9,70]. Our results also indicated that several compounds including avicularin, guajaverin, oleanolic acid and ursolic acid at some concentrations significantly increased the lysozyme activity in HKLs indicating that these compounds could be at least in part responsible for the effects observed for *P. guajava* crude extract and some fractions.

P. amarus extract with and without tannins as well as the hexane fraction were shown here to contain hypophyllathin which is identified as a major active compound from this plant. We show here that this compound may perhaps be responsible for a part of the effect of *P. amarus* non-tannins fraction on lysozyme production by HKLs but that its concentration does not seem to be high enough in other extracts to show activity. Other compounds which were not identified in this work should be responsible for the effects of the aqueous fraction.

HKLs contain lymphocytes, monocytes/macrophages and granulocytes (neutrophils, eosinophils, mast cells and basophils) [71]. Moreover, ROS, NOS and lysozyme are mainly produced in monocytes/macrophages or granulocytes [72]. In the present study, the increase of ROS, NOS and lysozyme activities in HKLs stimulated with the high dose of *P. guajava* ethyl acetate and dichloromethane fractions were accompanied with the increase of viability of HK cells at 24h. In contrast, these immune parameters did not consistently increase in parallel with the viability increase of cells treated with the *P. amarus* ethyl acetate and aqueous fractions. Moreover, *P. amarus* ethyl acetate and aqueous fractions significantly inhibited the NOS production in HKLs at some concentrations. This may be due to the fact that ethyl acetate and aqueous fractions of *P. amarus* could enhance the lymphocytes proliferation, which are mainly involved in the acquired or antigen-specific immune response but not on ROS, NOS and lysozyme production [73]. In agreement with our results, *P. niruri* aqueous extract (12.5–200 μg/ml) significantly enhanced the proliferation of T- and B-lymphocytes via the activation of MAPKs, whereas the *P. niruri* extract inhibited NOS production by bone marrow macrophages from treated mice [74]. Zuohua et al. (1994) showed the relationship between NOS production and T cell proliferation, when the decrease of NOS production was followed by the increase of lymphocyte proliferation in spleen cells after being stimulated with garlic derived diallyl trisulfide [75].

According to David et al. [15], flavonoids were identified as possible compounds responsible for the effects of the aqueous leaves extract of *P.*

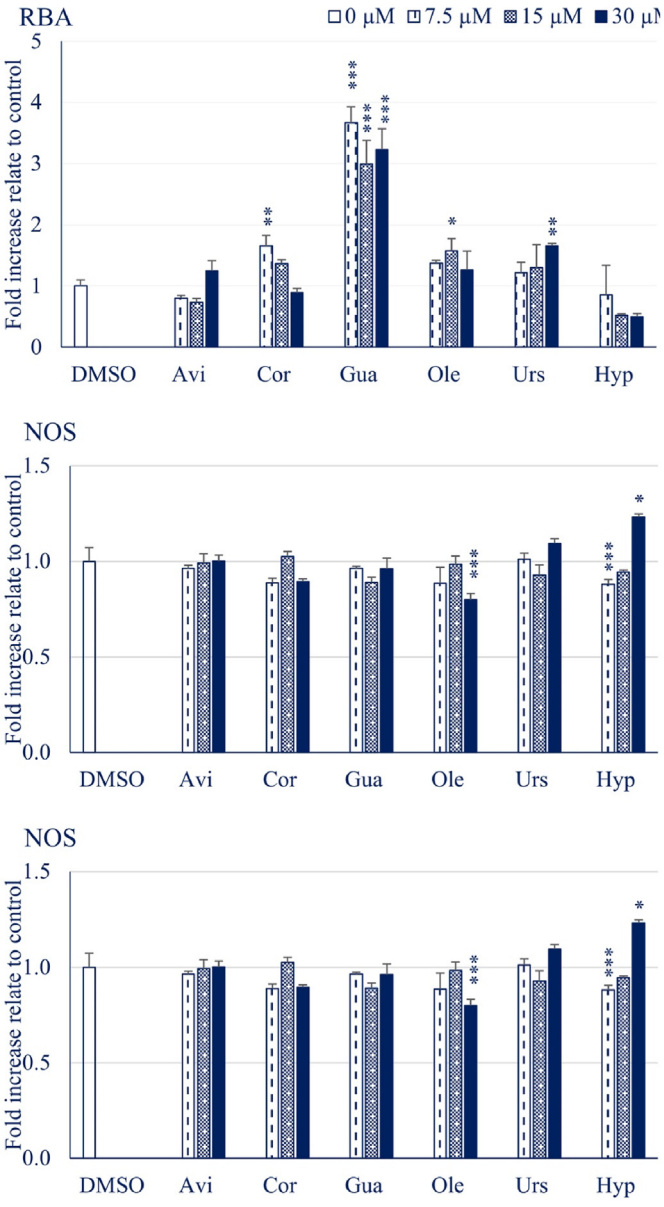

Fig. 13. Effect of pure compounds isolated from ethanolic extracts of *P. amarus* and *P. guajava* on RBA, NOS and lysozyme activities in striped catfish HKLs at 24h. Cor: corosolic acid; Gua: guajaverin; Urs: ursolic acid; Hyp: hypophyllanthin; Avi: avicularin and Ole: oleanolic acid. Asterisk indicates significant differences in lysozyme levels between stimulated and non-stimulated cells at 24 h (*$p < 0.05$; **$p < 0.01$; *** < 0.001). Values are mean ± S.D. (n = 3).

guajava that improved the immune system of the common carp, however there was no experiment carried out to test the flavonoid fraction nor pure compounds. Our researches demonstrated that not only the presence of flavonoids, but also triterpenic acids may explain at least partially the immunomodulatory effects of the dichloromethane and ethyl acetate fractions from the leaves extract of *P. guajava*.

Our findings revealed how *P. amarus* and *P. guajava* crude ethanol extracts, their fractions and pure compounds differently acted on the immune-regulation of striped catfish HKLs. However, the related literature regarding the *in vivo* application of any *P. amarus* or *P. guajava* fractions, as well as their pure compounds to improve the immune system of aquatic species is limited. Only the study by Sundaram et al. (2016) has demonstrated that acetone and petroleum ether extracts of *P. amarus* could protect the freshwater crab *Paratelphusa hydrodomous*

against white spot syndrome virus [76]. In parallel, many scientific papers demonstrated that *P. guajava* crude ethanol extract has successfully enhanced immune responses and defense mechanisms in striped catfish [22,23], rohu [12,14], tilapia [13]. These results suggested that the *P. amarus* and *P. guajava* crude extracts could be easily applied as a food additive to strengthen the immune responses and protect fish against pathogen.

5. Conclusions

The results indicate that *P. guajava* and its fractions, especially dichloromethane and ethyl acetate fractions, shown to contain triterpenic derivatives (both of them) and flavonoids (ethyl acetate fraction) differentially enhanced the RBA and NOS production as well as the lysozyme activity in striped catfish HKLs. On the other hand, several fractions of *P. amarus* including ethyl acetate and aqueous fractions inhibited the NOS production, responsible for the anti-inflammatory effect in HKLs. Compounds including corosolic acid, guajaverin, avicularin, oleanolic acid, and ursolic acid positively enhanced the RBA production and the lysozyme activity in dose and compound dependent manners. It could be concluded that the crude ethanol extracts of *P. guajava* and *P. amarus*, their fractions and pure compounds at certain concentration can potentially act as immunomodulators in striped catfish and could be considered as positive candidates in fishery sciences.

CRediT authorship contribution statement

Truong Quynh Nhu: Investigation, Formal analysis, Validation, Writing - original draft. **Nguyen Phuc Dam:** Investigation, Formal analysis, Validation, Writing - original draft. **Bui Thi Bich Hang:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Le Thi Bach:** Resources. **Do Thi Thanh Huong:** Conceptualization, Methodology. **Bui Thi Buu Hue:** Conceptualization, Methodology. **Marie-Louise Scippo:** Conceptualization, Methodology. **Nguyen Thanh Phuong:** Conceptualization, Methodology. **Joëlle Quetin-Leclercq:** Conceptualization, Methodology, Writing - review & editing. **Patrick Kestemont:** Conceptualization, Methodology, Writing - review & editing, Supervision.

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